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The influence of hydrogel density and RGD in cartilage tissue engineering

INAUGURAL-DISSERTATION
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1. Abstract

There is a worldwide interest to reconstruct functional articular cartilage tissue that has been lost due to injury or disease. Lesions in the articular cartilage are frequent as each year millions of people suffer from joint injuries, which cost billions of dollars for economy and society. Although mistaken as a disease impairing only elderly persons, often young people are affected, who are considered too young to undergo artificial joint replacement surgery. Articular cartilage has a very limited capacity of self-regeneration following trauma, injury, overuse or disease, which is due to its avascular nature and the limited mobility of the chondrocytes inside the compact cartilaginous matrix. A loss of cartilaginous tissue after joint damage therefore is irreversible.

Current treatment is limited to fighting the symptoms, such as pain and inflammation. Therefore many different scaffold constructs for articular cartilage repair have been thoroughly investigated over the past two decades. Various three-dimensional (3D) scaffolds have been evaluated, including alginate, hyaluronan, polyactic and polyglycolic acid, polyethylene glycol (PEG), agarose, fibrin and collagen, which aim to simulate and provide a cartilage-like environment to support proper chondrocyte growth and function. (for review see Hunziker et al., 2002) [1]

The aim of this study was to better understand chondrocyte interactions with their pericellular matrix, therefore we incorporated chondrocytes isolated from bovine condylar cartilage in 3D PEG hydrogels with different densities, some containing RGD or collagen type I. To examine cell response, we performed Live/Dead assay, histological and gene expression analysis for collagen type I and II, Sox9 and aggrecan. Chondrocyte viability was maintained within the hydrogels over a culture period of five weeks.

This study demonstrates that constructs with a density from 1.5% to 3.5% showed an increase in matrix production (ECM) and cell proliferation, whereas on the other hand, hydrogels containing RGD or collagen type I, had no positive effect on EMC production or cell proliferation.

2. Introduction

Cartilage is a highly specialized connective tissue consisting of round chondrocytes that organize the collagens, proteoglycans, sulfated glycosaminoglycans (sGAG, keratin sulfate and chondroitin sulfate) and non-collagenous proteins into an organized extracellular matrix (ECM) (Buckwalter and Mankin, 1998). [2]

To be able to work with cartilage, it is essential to understand the process of chondrogenesis and endochondral ossification. Natural chondrogenesis begins with the condensation and proliferation of mesenchymal cells (MC), at different time points during embryonic development. During mesenchymal condensation, the cells aggregate and begin to produce collagen I, fibronectin and proteoglycans. [3]

The aggregated cells then immediately begin to differentiate into chondroblasts.

Condensed mesenchymal cells start expressing Sox9, a transcription factor that itself controls downstream genes such as Sox5, Sox6 and collagen II α1, promoting progenitor cells to secrete cartilage-specific ECM molecules. [4, 5]

The three main types of cartilage, hyaline cartilage, elastic cartilage and fibrocartilage, vary in the composition of different types of collagen, fibrils and elastic fibers. (Young et al. 2000; Kirkham and Samarasinghe, 2009) While the predominant collagen in fibrocartilage is type I collagen, the predominant collagen in articular, hyaline cartilage is type II collagen. The collagenous framework is responsible for the form and the necessary firmness of the cartilage tissue. About 90% of the collagen in articular cartilage is type II collagen, which consists of three identical alpha-1 (II) chains, which are arranged in a triple helix. In 1971, the group around Hjertquist and Lamperg [6] showed in in-vivo studies on adult animals that cartilage defects heal with the formation of fibrocartilaginous repair tissue. It was shown that this repair tissue contains relatively low amounts of type II collagen and aggrecan which are functionally important markers of the chondrocyte phenotype (Benya and Shaffer, 1982) [7] and a relatively high amount of type I collagen, which can't be found in measurable amounts in normal healthy adult articular cartilage. With regards to the attempts of different tissue engineering methods to regenerate hyaline cartilage, it's essential to differ between hyaline like tissue and other connective tissues referred as "hyaline like cartilage" and "fibrocartilage" in the literature. The limited reparative capacity and poor healing of hyaline cartilage is due to its particular structure [8]

The most common proteoglycan found in cartilage is aggrecan, consisting of glycosaminoglycans, chondroitin sulfate, keratin sulfate and other sulfated polysaccharide chains. These side chains allow aggrecan to bind large amounts of water, which makes it resistible to compression during loading. In adult human and bovine cartilage 50-80% of GAGs are chondroitin sulfate (CS) chains. Several studies have shown that GAGs in adult human and bovine cartilage differ in their chain length, composition and sulfation as a function of development, age and disease. (Buckwalter et al., 1994; Lauder et al., 1998; Bayliss et al., 1999; Bayliss et al., 2000; Plaas et al., 2001; Sharma et al., 2007) It has been shown that the addition of HA to hydrogels or to a cell medium significantly increases cell proliferation and enhances the production of GAGs and collagen II. [9-12]

By applying mechanical forces to cartilage, a pressure gradient is generated in the interstitial fluid, causing fluid flow, referred to as the hydraulic permeability. Because of the limited pore size and the interaction between the negatively charged GAGs and the water molecules, a flow resistance is formed, limiting the fluid flow thought the ECM. [13]

During loading, synovial liquid is pressed into the synovial cavity of the joint, where the absorption of nutrients and the exchange of O_2 and CO_2 takes place. Thus, loading is essential for the nutrition and therefore for the function of cartilage. Another important glycoprotein is chondronectin, which mediates the attachment of chondrocytes to type II collagen. (Junqueira et al., 1996) [14]

At the edge of hyaline cartilage, in the perichondral zone, chondrocytes are more of an ellipsoidal shape, whereas chondrocytes from the inner zone of the cartilage are round and exist in groups of up to eight cells. (Junqueira et al., 1996) [14] Fully differentiated chondrocytes synthesize type II collagen, proteoglycans, hyaluronic acid and chondronectin. The differentiation phase of chondrocytes is accompanied by a change in cell morphology and the expression of specific markers. Each stage expresses different collagens, like type II, VI, IX, X and XI collagen. Type VI collagen is a marker protein for early proliferative stages of chondrocyte differentiation, whereas type X collagen is a marker for hypertrophic stage of late chondrocyte differentiation, which occurs physiologically during endochondral ossification.

In native tissue, chondrocytes maintain their spherical morphology and have the ability to produce cartilage-specific extracellular matrix (ECM), such as Aggrecan,

type II collagen and type I collagen. When cultured in monolayer as in a two dimensional substratum, chondrocytes tend to dedifferentiate into ellipsoidal shaped fibroblast-like cells and lose their ability to produce ECM. During this process the expression of type I collagen increases and the expression of type II collagen and aggrecan decreases. [15]

With extended passaging, dedifferentiated chondrocytes lose the ability to form cartilaginous tissue and the cells now tend to form fibro-cartilaginous tissue biomechanically inferior to the original hyaline cartilage (Schnabel et al., 2002) [16]. The pathways underlying dedifferentiation are not completely understood and characterized and can involve regression to any earlier developmental state, progression into a post differentiated senescent hypertrophic or pathological state, or conversion into a maintenance state that lacks features of the differentiated state. Attempts have been made lately to determine the pattern of gene expression that distinguishes differentiated articular chondrocytes from other types of chondrocytes, such as dedifferentiated cells and was shown in a study by Cheng T. et al., 2011. [17]
These facts led to a forced search for a different treatment approach of cartilage defects. During the last years, clinical approaches have been various, including microfracture, osteochondral allograft transplantation, autologous cartilage transplantation (ACT), autologous chondrocyte implantation (ACI), and many others. Autologous chondrocyte implantation has first been used clinically in the 1990s, after successful in vivo testing in rabbits. [18]. These first encouraging results led to the development of two fields of research in tissue engineering. The first field of tissue engineering research aims to improve the ACI technique by selecting the adequate autologous chondrogenic cells, the second field aims to develop three-dimensional matrices, capable of supporting the growth of phenotypically stable chondrocytes.
Various studies have incorporated biological signals into scaffolds and have evaluated their effects on cell behavior such as proliferation, ECM production, spreading and adhesion. The effects of the cell adhesion peptide RGD (arginine-glycine-aspartate) have been extensively studied for example by Mann et al. [19] Rowley et al. [20] and many others [21-23] As well as the effects of integrins which are heterodimeric cell surface receptors, mediate adhesion between chondrocytes and the ECM, by binding to ligands with an exposed RGD (Arg-Gly-Asp) sequence. These receptors stimulate intracellular signaling and gene expression involved in cell growth, migration, and survival. The same process, were found to lead to thrombosis,

inflammation, and cancer if not properly regulated. Because of this, research has focused on developing RGD peptides that would mimic cell adhesion proteins and bind to the cell surface receptors.

Research groups around the world compose hydrogels of different type of polymers, add biofactors, such as growth factors or even viruses, apply loading, all aiming to optimize the physical properties of the scaffolds.

Naturally derived polymers, such as alginate, agarose, chitosan, collagen - including collagen type I/II, fibrin and hyaluronan have been shown to be attractive biomaterials because of their biochemical similarity to cartilage. [24]

Collagen hydrogels promote cartilage formation by encapsulated chondrocytes. The cells interact with collagen gels via integrins, which can remodel collagen through the secretion of collagenase. [25] Hydrogels composed of collagen type II on the other hand, have been shown to promote efficient chondrogenic differentiation of embedded mesenchymal cells. [26] Currently the groups around Hao et al. and Hoemann et al. investigate the capability of cartilage defects repair in vivo, with chondrocytes encapsulated in injectable chitosan hydrogels and hydrogels based on chitosan and glycerol phosphate in phase II clinical trials. [27] [28]

3. Material and methods

3.1. Cartilage sampling

Full thickness bovine articular cartilage samples were obtained from the femoral head and the patellar surface from 6 Brown Swiss or Red Holstein cattle within 12hours post-mortem. The samples derived from cows of about 2 years of age. We had no information about a clinical history of arthritis or other pathology affecting cartilage, but in all samples there were lesions found on the cartilage surface, such as signs of fibrillation and change in color. All samples therefore were collected from areas which appeared normal by morphological examination.

The cartilage samples were immediately further processed, with the exception of the samples of native cartilage for PCR, which were kept in culture medium and were immediately frozen.

3.2. Preparation of media

3.2.1 Isolation Medium

Sterile DMEM-F-12, 50µg/ml gentamicin, 360 µg/ml L-glutamine

3.2.2 Culture medium

Sterile DMEM-F-12, 50µg/ml gentamicin, 360 µg/ml (2.5mM) L-glutamine, usually with 10% FBS or serum-free culture medium as indicated in the text.

3.3 Chondrocytes isolation

Cartilage was sequentially digested with 0.2% pronase and 2x collagenase P according to a previously described procedure. [29]

Briefly, isolation medium was pre-warmed to 37°C and then the total amount of Pronase enzyme needed was added. The cartilage then was dissolved for 60 minutes in a 37°C, 5% CO_2 tissue culture incubator. After Pronase digestion, the pronase was removed and the cell suspension was washed 3 times, 2 minutes each, with isolation medium. Then the suspension was filtered through a 0.22µm sterile Millipore filter.

The digestion was then continued by adding previously warmed collagenase P in isolation medium (final concentration is 0.0025%) and again dissolved for 60 minutes at 37°C. The resulting cell suspension was filtered through a 0.22µm sterile Millipore filter and then centrifuged at 1100 rpm for 15 minutes at 4°C. After a first centrifugation, the cells were washed in isolation medium and again centrifuged for another 3 times. After the last centrifugation, the chondrocytes were resuspended in 10ml isolation medium, counted, and either seeded in tissue culture flasks and cultured or frozen for later use.

3.4 Monolayer Culture

Chondrocytes were seeded in tissue culture flasks for cell expansion at a concentration of 2.5 x 10^6 cells per 150cm^2 flasks with a total volume of 20ml/flask.

Media was changed every other day until the cells reached confluence after about 1.5 weeks. At confluence cells were harvested by incubation with trypsin for 1 minute. Trypsin activity was neutralized by rinsing the cells with culture medium two times. The suspension then was centrifuged at 800 rpm for 5 minutes at 20°C. After the first centrifugation, the pellet was resuspended in 10ml culture medium and again centrifuged at 1400 rpm for 4 minutes. After this process the cells were either cultured for 7days and later frozen for gene expression or induced in hydrogels.

3.5 PEG hydrogel preparation

PEG hydrogels were formed as described earlier by Lutolf et al., Ehrbar et al. [30, 31] and Bryant et al. [32]. Hydrogel networks were formed by factor XIIIa crosslinking of n-PEG-Gln with n-PEG-MMP-Lys. The cross-linking reaction was performed in Tris-Buffer (TBS, 50mM, pH 7.6) containing 50mM calcium chloride and 10U/mL thrombin-activated factor XIIIa. For the formation of hydrogel disks, drops of yet liquid reaction mixture (20–40 mL) were sandwiched between sterile hydrophobic glass microscopy slides (obtained by treatment with SigmaCote, Sigma) separated by spacers of about 1mm thickness and clamped with binder clips. Prior to gelation, the drop of reaction mixture contacting only hydrophobic surfaces spread spontaneously to form a disc.

Hydrogels prepared at different precursor concentrations were formed by diluting concentrated 8-PEG-Gln/8-PEG-MMP-Lys precursor stock solutions (for 10% w/v gels) in order to minimize weighing error. Although gelation occurred within a few minutes at 37°C in a humidified incubator, the cross-linking reaction was allowed to proceed for about 1 h to ensure complete cross-linking.

A spare volume of the total reaction volume was used for the potential incorporation of the cell adhesion peptide RGD (Arg-Gly-Asp). A change in the crosslinking density of PEG hydrogels can be achieved by in-/ or decreasing the concentration of the PEG solutions, or by using branched PEG structures. [32-34]. A total of six different gel conditions were prepared. The conditions were: +RGD, - RGD, Col I, 1.5%, 2.5% and 3.5% crosslinking gel density.

3.6 Analysis of cell viability

To visualize the encapsulated chondrocytes in the hydrogels, a fluorescent membrane integrity assay, LIVE/DEAD® Assay (Invitrogen) was employed. All six different conditions were analyzed on day 0, 3, 7, 13, 17, 21 and day 38.
The 3D gels were cut in half so one part could be analyzed whereas the other half was further kept in medium for direct comparison of the same gel at different time points.

The hydrogels were rinsed with PBS for a few seconds, and 1ml of culture medium was added after the removal of the PBS. Then the constructs were stained with 1µl of 4mM calcein acetoxymethyl (Fluka) and 1µl of 2mM ethidium homodimer-1 (Fluka) and incubated for 10 minutes at 37°C.
After the incubation, the medium containing the dyes was removed and fresh culture medium was added. The samples then were imaged by fluorescence microscopy, using a Zeiss Axiovert 200M.

3.7 Gene expression analysis

Total RNA was isolated from three hydrogel constructs (1.5%, 2.5%, 3.5%) and from 2D cell pellet from day 7 as well as fresh cartilage as a control. RNA extraction was performed with RNeasy Minikit following the details in the manufacturer's protocol (Qiagen, Valencia, CA).
The cDNA was synthesized by reverse transcription (RT) with Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA). Then quantitative RT-PCR was performed by combining the cDNA solution with reagents from a Taq PCR Master Mix kit (Qiagen, Valencia, CA), as well as oligonucleotide primers and probes that were created for the genes of interest (Sox9, Agg, type II collagen, and type I collagen) and the housekeeping gene control (GAPDH and 18S).

3.8 Histology

Three hydrogels (1.5%, 2.5%, 3.5% no RGD) were harvested for histological evaluation at day 35. The samples were fixed overnight in 4% paraformaldehyde at 4°C and then transferred to 70% ethanol until embedded in paraffin and then cut in 4 - 5µm thick sections, according to standard histological techniques.

The sections were then deparaffinized, rehydrated and stained with Fast green and safranin-T as well as Masson's Trichrome to assess cell morphology, collagen fibers, glycosaminoglycans (GAG) and proteoglycan (PG) synthesis.

4. Results

4.1 Cell viability

Prior to seeding, a good cell viability (>95%) was assessed, by Trypan blue exclusion. The cell viability, as evaluated by Live/Dead double staining (Fig.2), varied significantly depending upon the ECM environment of the seeded chondrocytes. Generally, the manipulation of the cells during the seeding process seemed to have a negative effect upon their viability, which was reflected in an initial decrease of the cell viability. A few days after seeding the number of viable cells increased again in all six conditions.

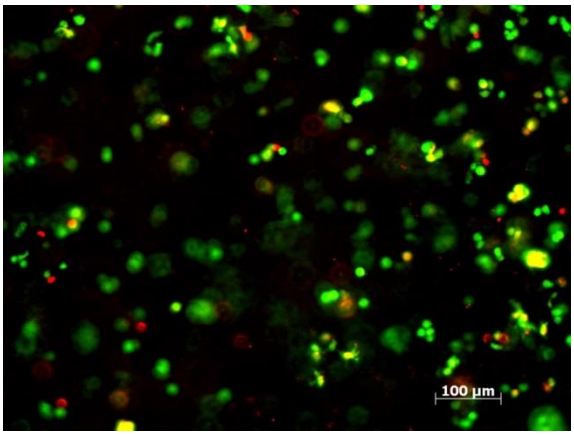
Cell viability within the +RGD, -RGD and Col I constructs initially significantly decreased between day 0 and 3, but steadily increased thereafter. In the other hydrogels the de/- and increase of viable cells could not have been observed as much.

The six different conditions were then compared to each other at 3 periods of time. The table (Fig. 1) further describes the observations that were made.

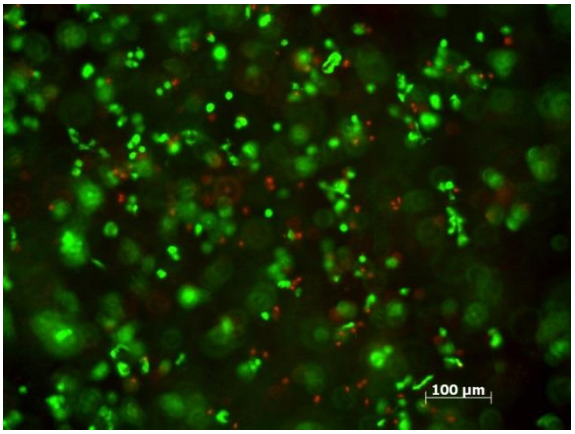
Fig. 1

Conditions	days 3-7	days 17-21	day 38
+RGD	• ~40% dead cells • Cells showed a rounded morphology • little ECM produced	• cell number increased • Cells showed a rounded morphology • ECM increased	• Very few dead cells • Cells showed a rounded morphology • ECM increased
-RGD	• ~40% dead cells • Cells showed a rounded morphology • Little but more ECM produced compared to +RGD	• Cell number increased • Cells showed a rounded morphology • Small clusters of 3-5 cells were formed	• Very few dead cells • Cells showed a rounded morphology • Cells are uniformly spread
1.5%	• Only a few dead cells • Cells showed a rounded morphology • Much ECM produced	• Only a few dead cells • Cells showed a fibroblastic morphology • ECM increased	• Very few dead cells • Cells are clustered in larger islands • ECM increased
2.5%	• Only a few dead cells • Cells showed a rounded morphology • Much ECM produced	• Very few dead cells • Small cell clusters • Cells showed a fibroblastic morphology • ECM increased, seemingly areas with more and some with less ECM (Lacunae)	• No dead cells found • Cells separated, no cell clusters • Cells showed a rounded morphology again, no fibroblastic appearance
3.5%	• More dead cells compared to 1.5% and 2.5% gels • Cells showed a rounded morphology	• Only a few dead cells • Small cell clusters • Cells showed a fibroblastic morphology • ECM increased	• Only a few dead cells • Cells showed a rounded morphology, little fibroblastic appearance • ECM increased, seemingly areas with more and some with less ECM (Lacunae)
Col I	• ~50% dead cells • Cells showed a rounded morphology • little ECM produced	• ~10% dead cells • Cells showed a rounded morphology • ECM increased	• Very few dead cells • Cells showed a rounded morphology • ECM increased

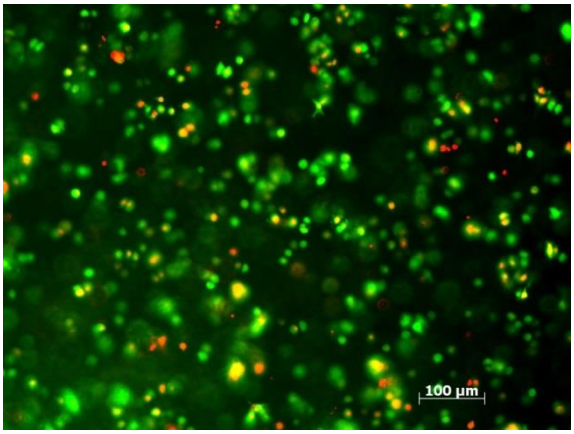
Fig. 2 Zeiss Axiovert 200M 10x magnification



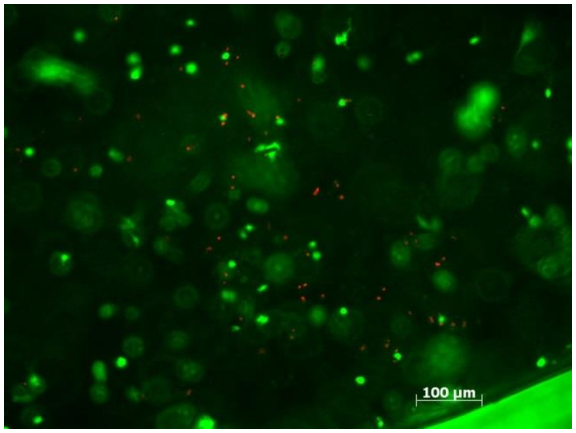
-RGD day 7



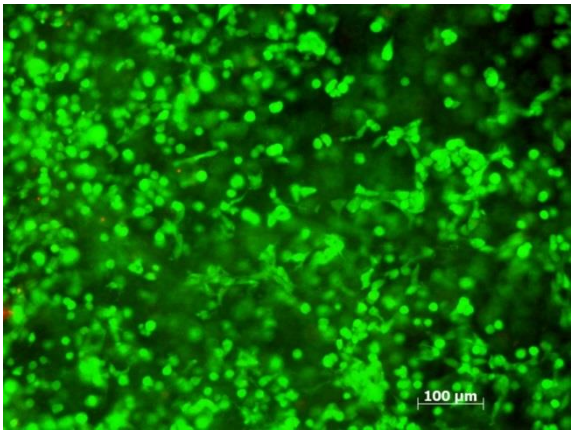
-RGD day 38



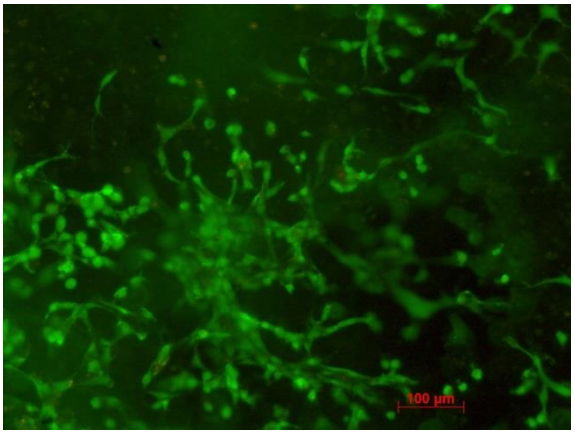
+ RGD day 7



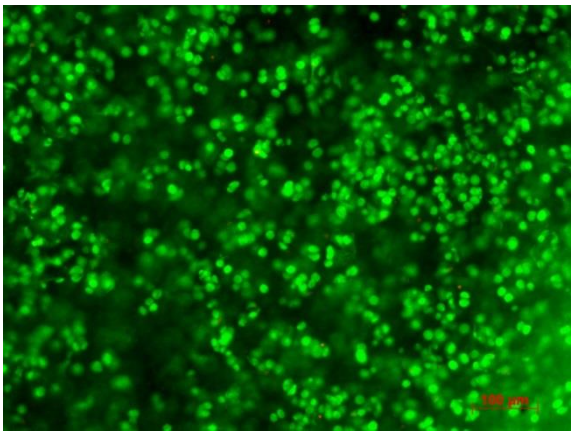
+ RGD day 38



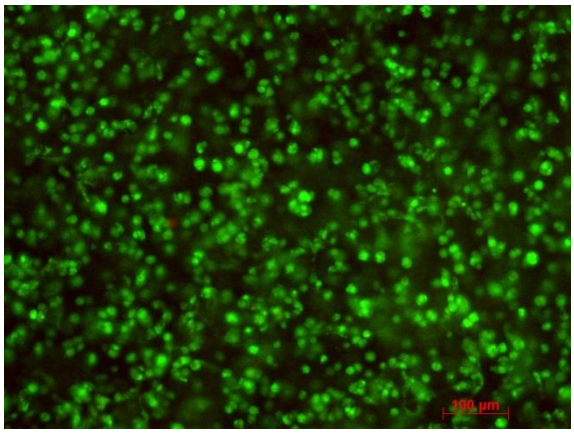
1.5% day 7



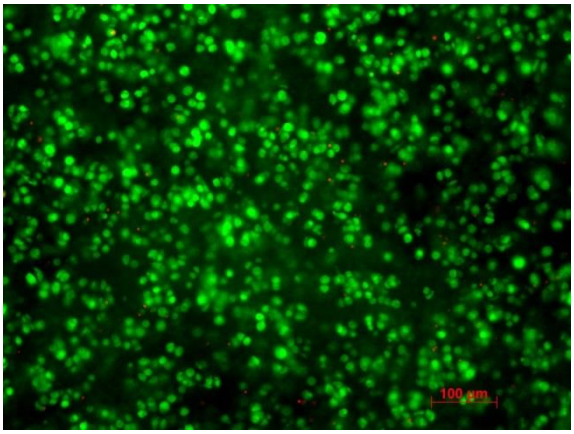
1.5% day 38



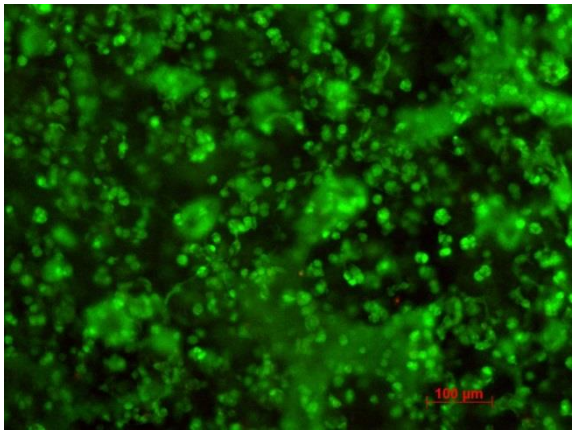
2.5% day 7



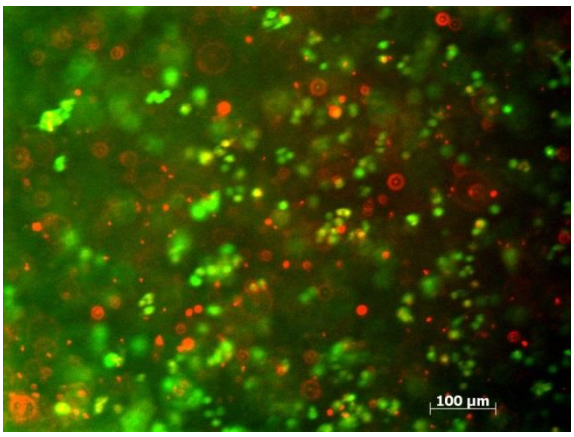
2.5% day 38



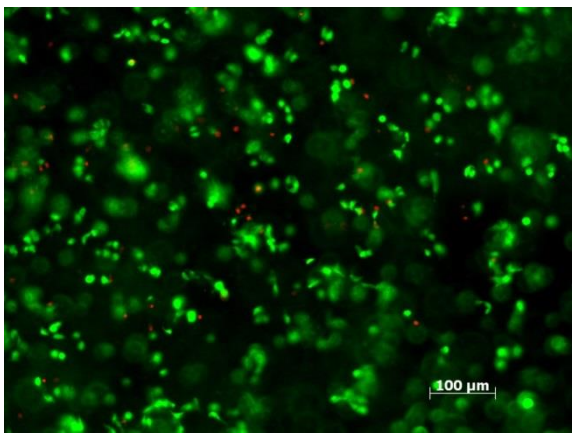
3.5% day 7



3.5% day 38



Col I day 7



Col I day 38

4.2 Gene expression analysis

To elucidate the effects of the different gel densities (1.5%, 2.5%, 3.5%) on gene expression, aggrecan, collagen type I, collagen type II as well as Sox9 expressions by chondrocytes encapsulated in the hydrogels, were normalized to the expression levels for native articular cartilage.

Collagen type I which is the most abundant collagen in the human body is believed to be expressed in dedifferentiated chondrocytes, whereas Sox9 is a key transcription factor associated with chondrocyte differentiation. Aggrecan which provides the osmotic properties necessary to resist compressive load, plays an important role in mediating chondrocyte-chondrocyte and chondrocyte-matrix interactions through its ability to bind Hyaluronan. [35, 36]

As shown in Figure 3, 4 & 5, the relative gene expression level of collagen type I increased in all conditions with the increase of culture time and it shows its maximum in the hydrogel with 3.5% density. Instead, the relative gene expression level of collagen type II decreased. Aggrecan first was up-regulated and then down-regulated in week 5. However, Sox9 shows a constant increase in the 1.5% and 2.5% density gels and a decrease after an initial increase in week 1 in the 3.5% gel.

These results indicate that the dedifferentiation was enhanced by the increase of the culture time and density.

However, the hydrogel with 1.5% density showed the best Collagen typ I/Collagen type II/Sox9 ratio as for non-dedifferentiated chondrocytes.

Fig. 3 Gene expression week 1

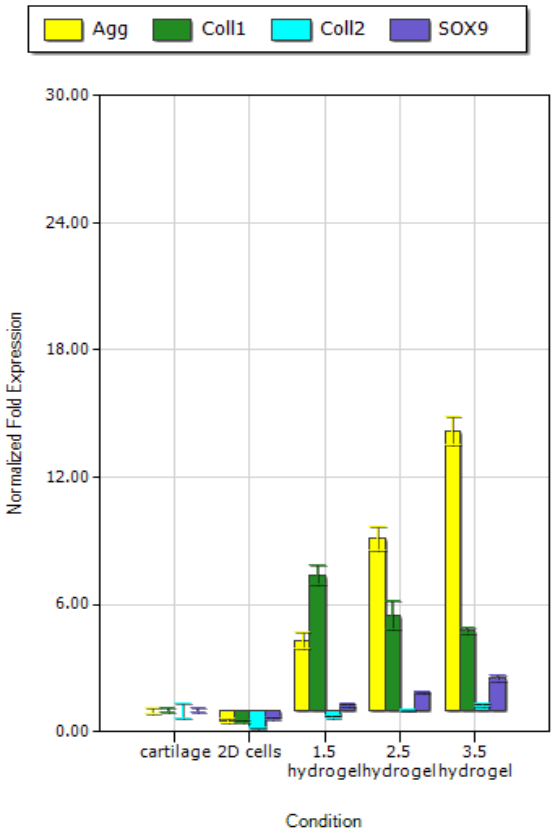


Fig. 4 Gene expression week 5

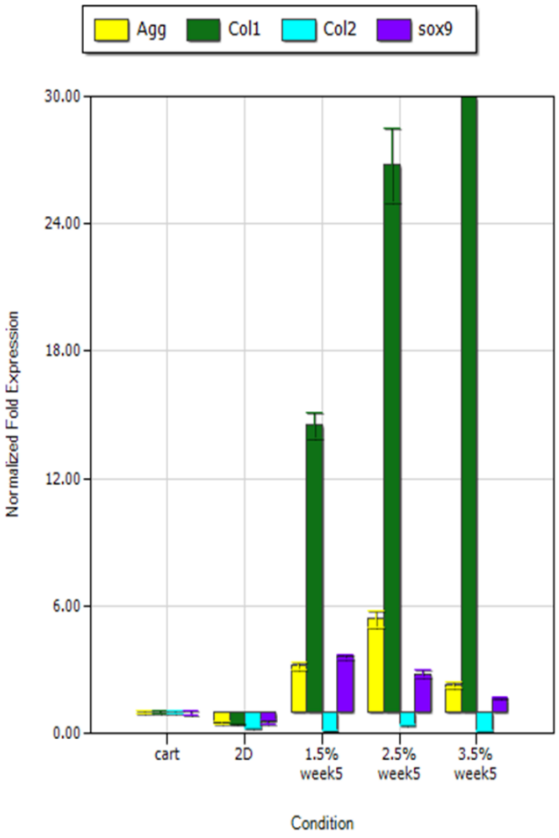
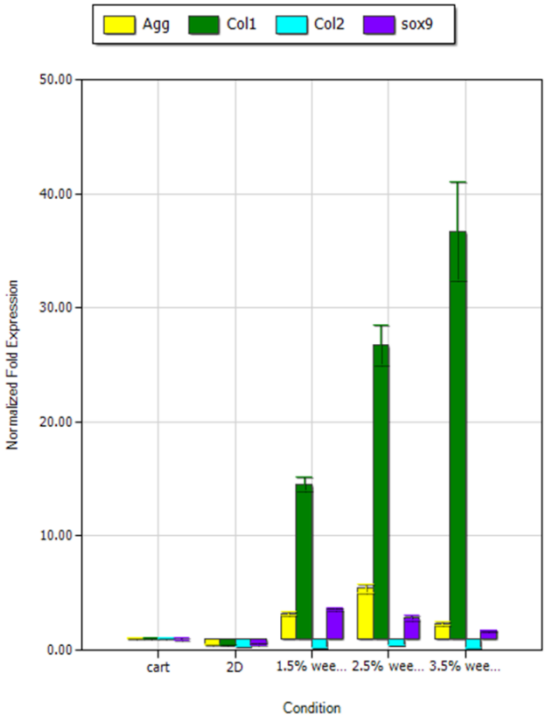


Fig. 5

Gene expression week 5,

Normalized Fold Expression to 50



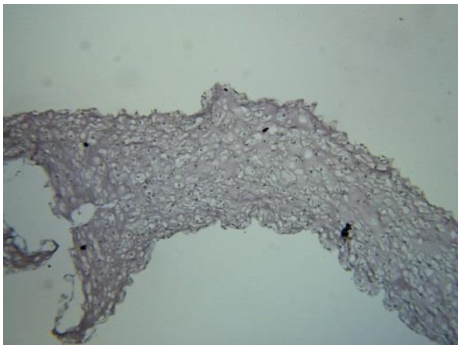
4.3 Histology

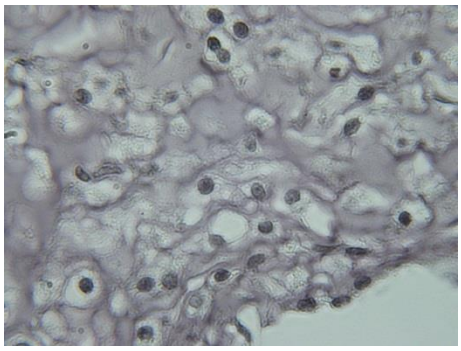
The histological evaluation confirmed the presence of a cartilage like tissue maturing within the hydrogels.

Figure 6 shows the staining of histological sections of 1.5%, 2.5% and 3.5% density hydrogels with Safranin-T/fast green for proteoglycans which are stained red and Masson's trichrome method for collagen which is stained blue, after 35 days of in vitro culture. Both, Safranin-T/fast Green and Masson's trichrome stained sections showed that the cells were evenly dispersed throughout the hydrogel beads.

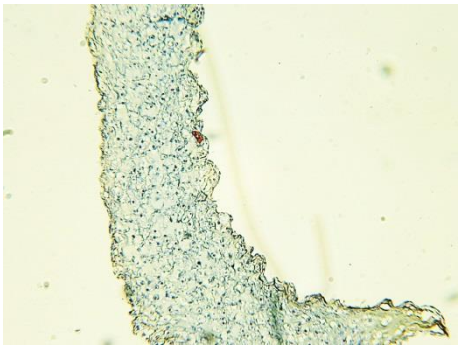
However, on the whole, the findings of the histological evaluation confirm the findings of the Live/dead assay. Generally the cells on the surface of the hydrogel constructs are smaller and less rounded than those in the interior. Overall, the cells in the 1.5% hydrogels showed less rounded and more fibroblastic morphology than the cells within the 2.5% and 3.5% hydrogels and the chondrocytes showed an elongated shape without lacunae. Also the more dedifferentiated appearing cells of the 1.5% gels formed a tissue with less Safranin-T/fast Green and Masson's trichrome staining. On the other hand the staining of the 2.5% and 3.5% gels indicate a high content of proteoglycans and collagen as confirmed earlier by gene expression.

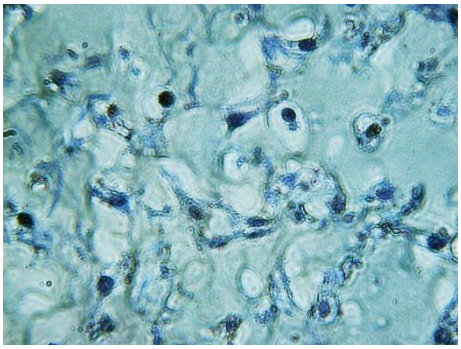
Fig. 6



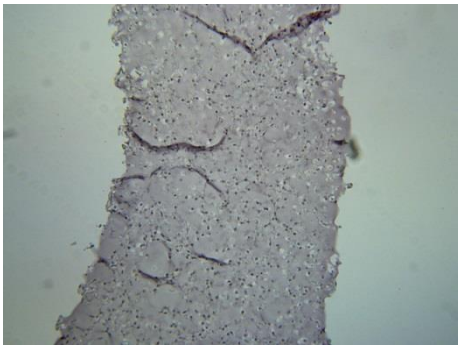


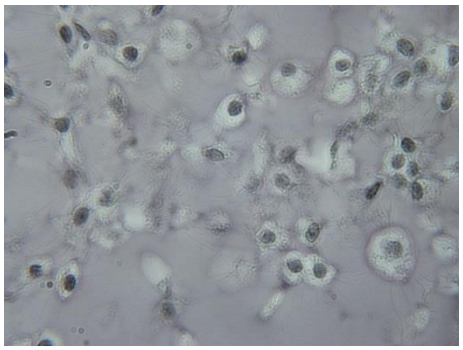
1.5% Safranin-T/ fast green





1.5% Masson‘s trichrome





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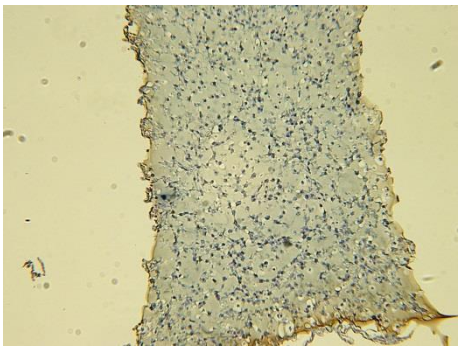

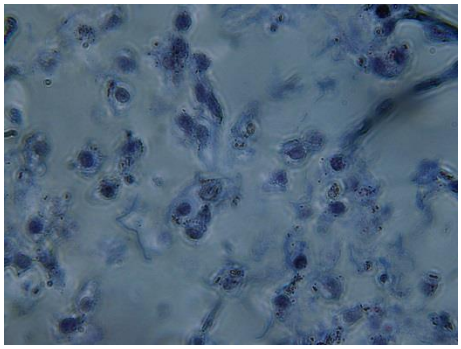

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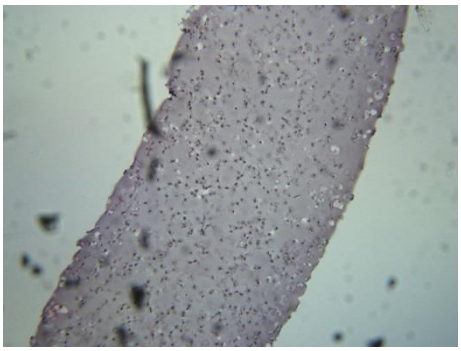
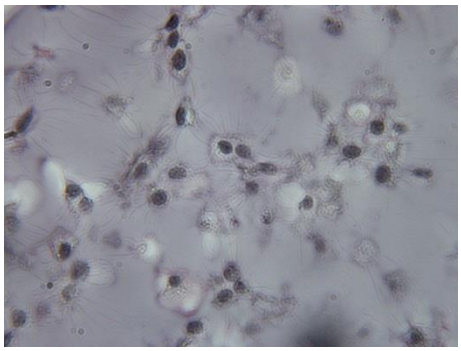
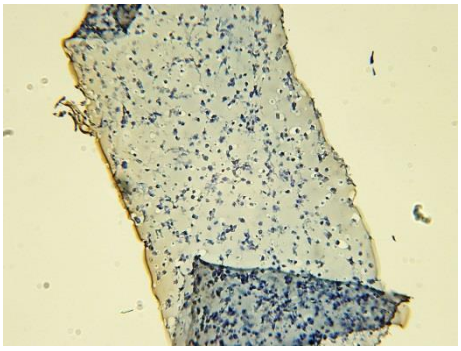
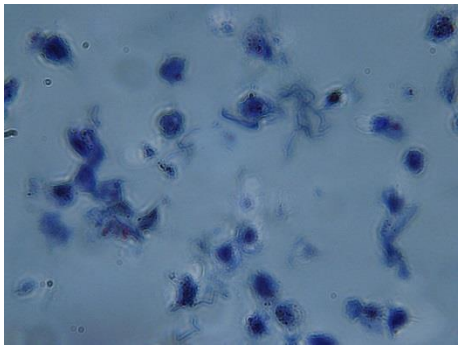




3.5% Safranin-T/ fast green





3.5% Masson's trichrome

5. Discussion

The goal of cartilage tissue engineering is to construct an implantable material with the aim to restore the original function by the regeneration of a fully differentiated tissue which has the same physiological properties as the healthy donor tissue. Although autologous chondrocytes implantation and second generation techniques based on the use of biocompatible and bioresorbable scaffolds preseeded with autologous chondrocytes have been widely applied in clinical practice for years, the results are far from being satisfactory as for a long-term therapy. In cartilage tissue engineering, the main problems are the production of a histological hyaline-like material, which can be fixed in the receiving site and provide mechanical stiffness without limiting the physiological function of a joint. The design of a 3D scaffold with the aim to induce and maintain cell differentiation, produce extracellular matrix (ECM) and ensure cell survival, requires an understanding of cell response in vivo and in vitro. It was shown that chondrocytes interact with their surrounding environment to receive biochemical cues from their ECM and PCM (pericellular matrix). [37] In 1993, Adams and Watt et al. showed that these interactions are essential in the regulation of tissue development and maintenance. Other studies investigating the chondrogenic differentiation of mesenchymal stem cells therefore suggest that fibronectin or its adhesive component, RGD are such needed cues to induce cell differentiation. [38]

The study presented here investigated the role of RGD and different densities in PEG hydrogels on chondrogenic differentiation. We chose the bovine model because of the accessibility of bovine tissue and because relatively large amounts of tissue can be obtained per joint. Also, bovine cartilage has been thoroughly investigated over the past years.

The cell viability as observed in the Live/Dead assay was good in all six constructs. In the overall comparison the gels without RGD and without Col I, showed significantly higher cell numbers, as well as viable cells, than gels containing RGD. The analysis of the six different gel conditions showed that the cells within gels with a density of 2.5% and 3.5% seemed to have the most alike morphology as compared to native cartilage and showed no or only little signs of dedifferentiation.

However, different research groups showed good results using collagen I gels containing autologous MSCs (mesenchymal stem cells) for in vivo studies on sheep [39] and in humans, where they have been transplanted into cartilage defects. [40]
In our study the constructs containing RGD did not stimulate cartilage repair sufficiently as shown in Live/Dead assay. Our findings confirm the results of previous studies made, which found no positive effects of RGD without the application of dynamic loading. [21, 41] Therefore, since the presence of RGD did not show the expected positive effects on chondrocyte proliferation, we decided to focus on the influence of different gel densities.
The findings of the Live/Dead assay were confirmed with histological analysis, which showed a more fibroblastic morphology of the cells within 1.5% hydrogels, compared to gels with higher (2.5% and 3.5%) densities. Also cells within hydrogels with a higher density seemed to produce more ECM compared to the gel with the lower 1.5% density, since a more intense Masson's trichrome and Safranin-T/fast Green staining could be observed.

Interestingly the comparison of gene expression analysis of the three different density conditions showed a better Agg, Sox9, Col2 to Col1 gene ratio in means of more differentiated chondrocytes in 1.5% density gels. Also, Sox9 was only constantly up regulated in the 1.5% gels, whereas it was only slightly up regulated in 2.5% and down regulated in 3.5% gels. These findings disagree with the observation of a more fibroblastic morphology and therefore more dedifferentiated appearance of the cells in Live/Dead assay and histological evaluation. This could mean that the differentiation process takes longer in lower density gels, but may result in a better gene distribution, compared to native cartilage. On the other hand, higher ECM production as seen in 2.5% and 3.5% hydrogels indicate a higher and faster cell proliferation.

Our findings are supported by previous findings of Bryant and Anseth et al. [34] where they described that chondrocytes produce more ECM components when they are encapsulated in PEG hydrogels with lower crosslinking densities and higher swelling ratios. These results were also confirmed by two groups working with MSCs instead of chondrocytes. [42, 43] Nicodemus et al. conclude that less crosslinked hydrogels may lead to the formation of a thicker pericellular matrix, since the ECM components may immediately surround the cells, which affects cell-matrix

interactions. [43] Kisiday et al. even concluded that in the absence of load, crosslinking density appears to be the dominant parameter that determines cartilage formation, because it regulates the diffusion of the ECM components. [44]

All these findings may be of interest for further investigation of the effect of different gel densities on chondrocyte proliferation. Longer culture times may be needed to detect changes in cellular response due to the change of the gel density as well as the addition of RGD, but in the presence of dynamic loading.

Overall we find it necessary to compare different conditions on different time points to the original donor cartilage by gene expression analysis. Only the direct comparison to native tissue allows an analysis of the differentiation process of the chondrocytes within the hydrogels. Histological staining as well as Live/Dead assay alone seems to leave too much room for personal interpretation. The use of histomorphometry, facilitating the comparison between stained sections, should be considered, which would help to compare the results more effectively.

Since chondrocyte behavior is controlled by multiple coefficients, combining different factors that have been found to be positively influencing chondrocyte behavior like dynamic loading, scaffold material, seeding density and growth factors, seems to be essential.

As shown by various research groups [45-47], the mechanical properties of cartilage depend on donor species and age and can vary widely. It was found, that immature chondrocytes tend to proliferate more and produce more ECM and show greater response to the application of growth factors, compared to mature chondrocytes. [48-50].

Therefor a potential limitation of our study may be due to different problems in context of harvesting of the cartilage material. In almost all donor animals, despite their age of approximately only two years, osteoarthritis (OA) could be found. As it was observed more often in knee joints than in hip joints, we would recommend harvesting native cartilage samples from hip joints, since the perimeter of the OA can’t be determined macroscopically and an influence on the surrounding ECM and collagen constructs can’t be excluded. Also the deepness of the cartilage collection regarding the total cartilage thickness at the harvesting sites should not be ignored, as different cell types could be found, due to enchondral differentiation. [51]

Although the exact mechanism of different hydrogel densities and the influence of RGD are still to be elucidated, this study demonstrates important findings of how

much small changes of the cell surrounding crosslinking density can change chondrocyte phenotype and ECM synthesis. Overall, our findings further support earlier observations and evidence in the literature, that the incorporation of RGD, without the presence of dynamic loading, does not show a positive effect on cell matrix interactions.

6. Summary and future research aspects

Current research has focused on creating three-dimensional (3D) scaffolds from biocompatible and biodegradable materials, which provide a structure and mechanical support for the cells seeded within the constructs, allowing them to ultimately growing into a functional tissue engineered construct.

Hydrogels have been demonstrated as suitable for creating scaffolds and they are well known for their biocompatibility. Recently, layered constructs were shown to be able to reduce strain discontinuities between the fabricated scaffolds and the surrounding natural cartilage. Thus, engineering functional cartilage out of artificial scaffolds with matching mechanical properties has proven to be extremely difficult and to make things even more challenging, the mechanical properties of the constructs change dynamically with time as the tissue regenerates and the scaffold degrades. Therefore, engineered cartilage so far shows to be mechanically inferior to native cartilage.

Although some research groups began to investigate the engineering of zonally organized cartilage, using different chondrocytes harvested from different zones of cartilage and encapsulating them in layered hydrogels [52, 53], there is still more understanding needed of how to construct cartilage with appropriately organized ECM. We also need to more investigate the degradation properties of the used scaffolds, such as hydrogels, because they determine the physical and structural properties of the scaffolds. Kloxin et al. therefor used a photodegradable nitro-benzyl-derived moiety, which they incorporated into PEG based hydrogels, making the degradation process visible by the application of light [41]. Little et al. [54] suggest to first develop a mechanical model, such as mathematical modeling, that may provide an alternative to mechanical testing. Another advantage of mathematical models is that it can be used to derive or estimate the mechanical properties of individual material phases within the cartilage. Different groups used the commercial software ABAQUS to model cartilage tissue behavior under compression. [55, 56]

Other popular approaches are methods and techniques to enhance the mechanical properties, by controlling the amount of ECM such as PG and collagen produced.

Hansen et al. and Waldman et al. showed that collagen and PG levels within the ECM can be controlled with hydrostatic pressure and dynamic shear or compression. [57, 58] Hansen et al. showed that a loading frequency of 1Hz is optimal for ECM

production, whereas the ECM production can be inhibited with low (0.3Hz) or high (3Hz) frequencies. It also has been shown that the compression amplitude can regulate collagen and PG synthesis. [59]

These and similar findings have been recently applied to bioreactor design, with the aim to mimic the dynamic forces seen in the native joints and thereby create cartilage with similar mechanical properties. [60, 61]

Commonly fetal bovine serum (FBS) is added to cell culture, since it is known to increases cell proliferation and ECM production [62] Recent studies now have used different individual stimulants, such as bone morphogenetic proteins (BMP) or transforming growth factors, which compared to FBS have more defined composition and therefor give more repeatable results. [63-65]

Also, more studies are required, investigating the effectiveness of chondrocyte sources. It is important that future studies concentrate on cells from clinically relevant sources, that don´t cause considerable damage to the donor site. Therefor MSCs are a valuable chondrocyte cell source because of ease with which they can be isolated and their various differentiation capabilities, but nevertheless that cell type still needs further investigation, since researcher are faced with challenging problems using MSCs in in vivo studies [66, 67]. However, an interesting finding about MSCs, which may be useful for clinical trials, is that it could been shown, that MSCs derived from skeletally mature horses produced superior cartilage tissue compared to age-matched chondrocytes in peptide hydrogels. In addition, it was also shown that MSCs proliferated in the presence of TGFβ1, whereas the mature chondrocytes did not [48]. Therefor the hydrogels used in studies using MSCs should release growth factors such as TGFβ1, since they can reduce the in vitro cultivation time before implantation. Interesting newer studies tried to combine a slowly degrading scaffold material with high bulk stiffness, such as PCL (poly ε-caprolactone) and TGFβ3 physically complexed with CS (chondroitin sulfate). They designed an interconnected pore network which lasts for at least 6 months, what allows the neo-cartilage formed from MSCs, to have sufficient time to mature without biomechanical overload. [68-70]

Further research with the goal of in vivo application must also address immunological issues, such as the integration of host and stem cell based constructs and their interaction with the surrounding tissue, its age, disease and physical activity.

Therefore, interdisciplinary studies are crucial to develop cartilage tissue engineering´s full potential in cartilage repair.

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8. List of used abbreviations

PEG	poly(ethylene glycol)
PBS	Phosphate buffered saline
ECM	extracellular matrix
PCM	pericellular matrix
HA	Hyaluronic acid
LP	link protein
AGC	Aggrecan
sGAG	sulfated Glykosaminoglycane
CS	Chondroitinsulfate
PS	polystyrene
RT-PCR	real time polymerase chain reaction
COL2	collagen type II
COL1	collagen type I
SZP	superficial zone protein
SOX9	SRY (sex determining region Y)-box 9
CLU	clusterin
MIA	melanoma inhibitory activity
COMP	cartilage oligomeric matrix protein